Effects of Metformin on the PI3K/AKT/FOXO1 Pathway in Anaplastic Thyroid Cancer Cell Lines

Running title: Effects of Metformin on PI3K/AKT/FoxO1 pathway in ATC

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Abstract

Background: The PI3K/AKT/FOXO signaling pathway plays an important role in the survival, proliferation and apoptosis of tumor cells. The aim of the present study was to explore whether metformin could affect insulin-promoting cell growth by regulation of this pathway. **Material and Methods:** Anaplastic thyroid cancer cells were treated with 0-60 mM metformin for 24, 48 and 72h. Cell viability, morphology, apoptosis and migration were investigated by MTT assay, microscopy observation, AnexinV-PI and the wound healing assay, respectively. Expression levels of PI3K, AKT and FOXO1 were detected by RT-qPCR, and proteins phosphorylated levels were determined by ELISA. **Results:** Metformin decreased cell viability and migration in a significant time-and dose-dependent manner, and induced apoptosis and morphological changes in the cells. RT-qPCR results showed that expression levels of PI3K, AKT and FOXO1 was inhibited by metformin ($P<0.05$). However, there was no significant change in the expression level of AKT following metformin treatment for C643 cell line ($P>0.05$). ELISA results showed that metformin treatment had no significant effects on the phosphorylated levels of PI3K, AKT and FOXO1 ($P>0.05$). **Conclusuion:** The downregulation of FOXO1 was intensified by metformin, but no increase in cell viability was observed following FOXO1 downregulation by metformin. However, the exact molecular mechanism of metformin on inhibition of the PI3K/AKT pathway and subsequent decrease in cell viability remains unclear and further studies are required for its clarification.

Key Words: Anaplastic Thyroid Cancer, Metformin, PI3K, AKT, FOXO1

Key Points:

- Metformin decrease cell viability of ATC-derived cells in a dose-and time-dependent manner
- Decrease of cell viability by metformin significantly associated with the downregulation of PI3K and AKT mRNA levels of the PI3K/AKT signaling pathway
- Metformin increased the downregulation of FOXO1but an increase in cell viability following FOXO1 downregulation was not observed

Introduction

Thyroid carcinoma is the most common malignancy among the endocrine neoplasms. Histopathologically, four main subtypes have been demonstrated for thyroid cancers, which differe in cellular origin. Thyroid follicular cells are the source of papillary (PTC, 85% of cases), follicular (FTC, 5–10% of cases) and undifferentiated or anaplastic (ATC, 1% of cases) subtypes; para-follicular cells (C-cells) are the origin of medullary (MTC, 5%) form [1, 2]. ATC is the most aggressive and malignant form of thyroid neoplasm and accounts for less than 2% of all thyroid cancers, although it is the cause of over 40% of deaths from thyroid cancer [3]. In general, ATC has a very poor prognosis with a 3- to 7-month survival rate [4]. Because of its aggressive nature, conducting studies on the treatment of ATC is difficult; conventional treatments involving a combination of chemotherapy, radioiodine therapy, external beam radiation, and surgery have rarely been effective or curative. Hence further investigation on the molecular pathways of dedifferentiation and identification of therapeutic targets and novel therapeutic strategies for ATC patients are needed. Currently, new molecular targets, new agents and their combinations are being investigated [3].

There is a growing body of epidemiological evidence showing a strong association between diabetes mellitus type 2 (T2D) and obesity, with a higher risk of several types of cancer including thyroid cancer. Furthermore, an association has also been reported between T2D and thyroid volume and the incidence of thyroid nodules; such associations have been attributed to the hyperglycemia and hyperinsulinemia resulting from the insulin resistance in these patients. Insulin growth-promoting effects are exerted through activation of insulin/insulin-like growth factor-1 (IGF-1) receptor-dependent signaling [5]. Metformin is a widely used anti-diabetic drug for the treatment of T2D [6]. It has been proposed that metformin has antineoplastic properties against a variety of cancers [7-10]. In thyroid cancer, a retrospective analysis of medical records of patients with DTC and type-2 diabetes mellitus simultaneously, showed increased survival in those who were treated with metformin [6]. Comparison of two PTC groups including metformin treated and non-treated groups, demonstrated that size of thyroid tumors was significantly lower in the metformin-treated group, suggesting that metformin has an inhibitory effect on tumor growth [11]. Furthermore, there are numerous studies demonstrating that metformin reduces cell proliferation in thyroid cancer cells including MTC, ATC, and PTC cell lines [12, 6, 13]. However, in thyroid cancer cells, the precise antitumor mechanisms of metformin and downstream effectors underlying its actions remain largely unclear [14]. Improvement of insulin resistance and reduction of serum insulin levels are possible mechanisms proposed for the inhibitory effects of metformin on human cancers, which could affect insulin/IGF-1 signaling-dependent cancer cell growth [15]. Phosphoinositide 3-kinase (PI3K)/ Protein kinase A (AKT) signaling appears to play an important role in ATC tumorogenesis [16]. AKT has a critical role in the several cellular processes including regulation of cell growth, proliferation and apoptosis and PI3K is the most important upstream molecule to phosphorylate and activate AKT [17]. Furthermore, forkhead box protein O1 (FOXO1) is a transcription factor of the FOXO family, which plays an important role in insulin and IGF-I signaling pathways and also in different cellular processes including cell prolifration, apoptosis, cell cycle and metabolism [18]. According to the FOXO1 roles in a wide range of cell processes and its downregulation in diffetent types of human tumors including breast cancer [19], endometrial cancer [20], Hodgkin's lymphoma [21] and prostate cancer [22], it has been suggested that FOXO1 may be involved in the carcinogenesis [23]. PI3K/AKT is the main regulatory pathway of FOXO1 protein. In summary, AKT phosphorylates FOXO1 at multiple sites and causes its accumulation in the cytoplasm. It also inhibits FOXO1 transcriptional functions which in turn causes cell growth and prolifration [24]. Although FOXO1 has been demonstrated as a novel tumor suppressor in various types of human cancers, its role in thyroid cancers has not been well established [25]. In the present study, the *in vitro* effects of metformin on the regulation and activation of PI3K/AKT/FOXO1 signaling pathway have been explored in ATC-derived cell lines. Furthermore, the effects of metformin on ATC cells properties that are required for development of metastasis were also examined.

Materials and Methods

Cell culture

Human ATC cell lines were purchased from thevIranian Biological Research Center (SW1736 and C643) and Pasteur Institute of Iran (8305C). Cells were cultured in RPMI-1640 (SW1736 and C643) or DMEM (8305C) medium supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100µg/ml streptomycin and 1% non-essential amino acid (for 8305C) at 37˚C and humidified 5% CO_2.

Human tissue

Human thyroid tumor samples kindly provided from patients underwent guitar surgery, samples were frozen immediately upon reception. All patients participating in the study gave their informed consent and protocols were approved by the institutional ethics committee.

Cell viability assay

In order to assess cell viability, the MTT (3-[4, 5-dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide) assay (Sigma Aldrich) was used. Different cells, after preculture in 96-well plates (5-6×10^3 cells/well) and achieving 60% confluence, were treated with increasing doses of metformin (Alborz Bulk Pharmaceutical company in Tehran, Iran), including 0-60 mM concentrations for 24, 48 and 72h. MTT assay was performed by incubating the cells with 0.5 mg/ml MTT for 3 h at 37˚C in 5% CO_2. The purple deposits of formazan was dissolved by DMSO (dimethyl Sulfoxide), and absorbance was monitored at 570nm and 630nm as the reference wavelength by a spectrophotometer. All experiments were repeated at least three times in sexaplicate and the IC_{50} of metformin was calculated for each time period.

Cell morphology Studies

To investigate morphological changes of the metformin-treated ATC cell lines, the cells (5-6 × 10^3 cells/well in 96-well plate) were treated with 0, 30 and 60mM metformin for 48h and the changes were monitored under an inverted microscope (Olympus CKX31; Olympus Corporation, Tokyo, Japan). These values were selected to comprise morphological results in different doses.

Cell migration assay

Different cell lines were plated in the appropriate number (1×10^5 cells/well in 24-well plate) for 100% confluence in 24 hours. In a sterile condition, a vertical wound was performed by a 100µl pipette tip through the cell monolayer; the media and cell debris were then aspirated and wells were washed by PBS. Enough medium with and without metformin were added to the wells and the cell culture plates were incubated at 37°C and 5% CO_2. At several time points, the plates were removed from the incubator to take a snapshot picture and to check for wound healing. All experiments were performed at least three times.

Flow cytometry

Briefly, cancer cells were plated in 6-well plates (1×10^6 cells/well) and treated by 0, 10 and 30 mM concentrations of metformin for 48h. After treatment; floating cells were collected from the original growth medium and combined with the trypsinized adherent cells. The assessment of apoptotic and necrotic cell death was performed using double staining with fluorescein Isothiocyanate-conjugated Annexin V-FITC and Propidium Iodide (PI). Annexin V binds to Phosphatidylserine residues of apoptotic cells, whereas PI labeles the necrotic cells with membrane damage. Detached cells were stained for 15 min with Annexin-FITC and PI using the Annexin V-FITC kit (Bio Legend, Cat. No. 420201, Canada). Staining was performed according to manufacturer instructions and stained cells were analyzed by FACS Calibur flow cytometer (BD Biosciences, Burlington, MA); the numbers of viable, apoptotic, and necrotic cells were calculated with Flowjo software (version. 10).

mRNA and protein extraction

In order to investigate gene expression, after treatment with different concentrations of metformin (0, 5, 10, 20 and 30mM), the cells were trypsinized, detached and collected into 1.5ml RNase/DNase free tubes. Total RNA and total protein were extracted according to manufacturer's instruction AllPrep DNA/RNA/Proitein Mini Kit (QIAGEN, Cat. No: 80004, Germany). RNA quantity and purity were determined by NanoDrop 1000 (Thermo Scientific, Waltham, and Mass).

Real-Time PCR

For each sample, 1µg total RNA, 1µl random hexamer and 10µl RT Pre-Mix were added to the 20µl RT reaction system. Complementary DNA (cDNA) was synthesized according to the manufacturer protocol (BioFact RT series, Korea). The primers were designed using GeneRunner software (version 4.0), cheked in NCBI Primer Blast and ordered to the Pishgam Company (Tehran, Iran), and their sequences were shown in Table 1. RT-qPCR amplification was performed in a 25-µl reaction by SYBR Green master mix (Thermo Fisher Scientific, USA). The RT-qPCR cycler used was Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia). The amplification conditions of the quantitative PCR were as follows: 95˚C for 10min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 20 sec, 72˚C for 40 sec. All experiments were performed at least three times. The expressions were normalized to b-actin mRNA, and the relative mRNA levels were determined using the $2^{-\Delta\Delta Ct}$ method.

Enzyme linked immunosorbent assay (ELISA)

Total extracted protein from all cells collected were analyzed by ELISA according to manufacturers instructions. ELISA kits for p-AKT (ZB-14054S-H9648), p-PI3K (ZB-14242S-H9648), and p-FoxO1 (ZB-14227S-H9648), were from ZellBio GmbH Germany, which are based on the sandwich method. The amount of total extracted protein was determined using the Bradford method.

Statistical analysis

Statistical analyses were performed with MedCalc 14.8.1 software. The normal distributed data was expressed as the mean ± SD. Statistical differences were considered significant when probability value was <0.05. Relative gene expression was assessed by relative expression software tool (REST, version 2009).

Results

Metformin inhibits growth of ATC cell lines

The growth inhibitory effects of metformin were investigated on anaplastic thyroid cancer cell lines, including SW1736, C643 and 8305C, and mean IC_{50} values in the 24-, 48- and 72-h treatments were calculated (Table.2). According to Fig. 1, metformin significantly decreased cell viability of the ATC cell lines in a dose- and time-dependent manner. Among different ATC cell lines, SW1736 and C643 cells were more sensitive and the growth-inhibitory effect on 8305C cells was not more significant; maximal effect of metformin was observed at 72-h incubation.

Metformin modifies morphology of the ATC cells

In addition to cell viability, metformin remarkably affected the morphology of ATC cell lines. Microscopic examination illustrated a significant decrease of cell density and morphological changes of metformin-treated cells including smaller, elongated, and granulated shapes (Fig. 2).

The effects of metformin on ATC cells migration

To analyze the results of cell migration assay, the area of the wound was measured by Image J software. Wound-healing assay results indicated that metformin-treated cells displayed decreased migration, compared with negative control cells. Bar graphs of the scratch assay experiment at different time points are presented in Fig 3.

Flow cytometry

Flow cytometry was applied to determine whether metformin-induced decrease in cell viability was accompanied by apoptosis. According to the results, metformin induced apoptosis in ATC cell lines; the most significant effect of metformin on cell apoptosis was observed in 30mM concentration in which metformin enhanced the percentage of apoptotic and necrotic cells from 8.78% to 16.65% in SW1736, from 12.77 to 42.9% in C643 and from 4.31 to 7.69% in 8305C cells. In 10mM concentration of metformin the increase of apoptotic and necrotic cells was as follows: in SW1736 cells from 8.78 to 10.16%, in C643 cells from 12.77 to 12.12%, in 8305C from 4.31 to 4.45% (Fig. 4).

FOXO1 expression is decreased in ATC-derived cell lines

Given that FOXO1 protein has been considered a possible tumor suppressor, its expression in ATC-derived cell lines was compared with the expression in human normal thyroid tissues [23]. To this end, RT-qPCR was performed with mRNAs isolated from a panel of six surgically removed normal human thyroid tissues from different patients. The mRNA from normal tissues (NT) was used as the control and FOXO1 mRNA expression in the ATC cell lines, compared with expression in NT. We found a critical decrease in FOXO1 expression levels in all cell lines examined ($P<0.05$) (Fig. 5).

Effects of metformin on PI3K, AKT and FOXO1 expression and protein phosphorylation

To measure PI3K, AKT and FOXO1 expression in ATC cell lines, quantitative RT-PCR was conducted on SW1736, C643 and 8305C cell lines after treatment with different concentrations of metformin. Quantitative RT-PCR showed that the expression of PI3K, AKT and FOXO1 decreased in the metformin-treated SW1736 cell line compared with the untreated cells as the negative control. In the C643 cell line a decrease was observed in both PI3K and FOXO1 mRNAs expression whereas the mRNA level of AKT was not significantly modulated by metformin, compared with negative control. A significant decrease was also observed in PI3K, AKT and FOXO1 mRNAs expression in the 8305C cell line (Fig. 6). To investigate metformin effect on phosphorylation status of PI3K, AKT and FOXO1 in ATC cell lines, enzyme-linked immunesorbant assay was used. Based on ELISA results, there was no significant modulation in the phosphorylation of PI3K, AKT and FOXO1 proteins in any of the examined cell lines following metformin treatment (Fig. 7, $P>0.05$).

Discussion

In the present study we investigated whether metformin could affect insulin-promoting cell growth by regulation of the PI3K/AKT/FOXO1 pathway. Thyroid cancer is the most common endocrine malignancy and ATC is the most aggressive form of not only thyroid but all of human cancers. We used ATC-derived cell lines for this mentioned aim. We used SW1736, C643 and 8305C cell lines to examine the effects of metformin on anaplastic thyroid cancer cell growth, apoptosis, migration and morphology. According to our results, metformin exerted inhibitory effects on ATC cells growth in a dose- and time-dependent manner. The strongest effect of metformin on the inhibition of cell growth was observed 24h after treatment. Growth inhibitory effect was associated with decrease in cell migration, microscopic morphological changes and apoptosis induction in cancer cells. According to the MTT assay and flow cytometry results of the examined cell lines, C643 and 8305C were respectively the most sensitive and resistant cell lines to metformin, findings which correspond to the higher growth velocity of the 8305C cell line.

According to our findings, the expression level of PI3K, AKT and FOXO1 was decreased by metformin ($P<0.05$), although there was no significant change in the mRNA expression of AKT in the C643 cell line ($P>0.05$). A critical down-regulation in FOXO1 was found in all examined cell lines, compared with normal thyroid tissues and metformin had no significant effects on the phosphorylated levels of PI3K, AKT and FOXO1 ($P>0.05$).

The vast majority of epidemiological studies report a well-documented association between T2D with increasing risk of different malignancies including thyroid neoplasia and insulin resistance following hyperglycemia and hyperinsulinemia has been introduced as the causality of this association. Insulin, a well-identified growth factor, exerts its growth-promoting effects via insulin/IGF-I signaling [5]. Recently, it has been reported that insulin has relevence to tumor growth and metastasis [26]. Furthermore both insulin and IGF-1 induce cell growth through stimulating the PI3K/AKT/FOXO signaling pathway. Consequently, PI3K/AKT-dependent phosphorylation of FOXOs facilitates their nuclear exclusion and eventual proteasomal degradation [27].

Because of different important roles in cellular processes, FOXO1, an important member of FOXO protein family, has been proposed as a novel tumor suppressor transcription factor that may be involved in tumorgenesis, this transcription factor is downregulated by the insulin-activated PI3K/AKT pathway [23]. According to the study performed by Song et al, the tumorigenesis of

PTC might be associated with down-regulation of FOXO1, which may have a critical role in inhibition of PTC development by regulating cellular proliferation, growth, and apoptosis; they introduced FOXO1 expression as a potentially useful biomarker for PTC [25]. In addition, Zhang et al. showed that FOXO1 plays an important role in inhibition of human cervical cancer development by cell cycle and apoptosis arrest [28]. However, as discussed above there is limited evidence on the relation of FOXO1 to thyroid cancers and its role in thyroid cancers has not been well established [25]. Zaballos et al. indicated that FOXO1 was differentially expressed in normal and tumor tissues in many human malignancies, including PTC [23]. They also demonstrated that, FOXO1 mRNA and protein levels were significantly reduced in different human thyroid cancer cell lines, compared with normal human thyroid tissues [23]. In the current study, we considered FOXO1 expression in ATC-derived cell lines and compared it with expression in human normal thyroid tissues. Like Zaballos's study, we also found a significant decrease in FOXO1 mRNA levels in all undifferentiated thyroid cancer cell lines.

Metformin increases glucose uptake by activating the AMP-activated protein kinase (AMPK) signaling pathway [29] and most of the studies investigating its antitumor effects have focused on the AMPK signaling pathway [30]. Previously, it has been demonstrated that constant activity of the PI3K/AKT pathway plays a determining role in the development of different types of cancers [31]. Therefore, researchers focusing on this pathway, as one of the metformin targets, found that in addition to the AMPK pathway, metformin affects cell proliferation by regulation of the PI3K/AKT signaling pathway [17]. In the present study, the analysis of the qRT-PCR data revealed that the mRNA expression level of PI3K was significantly decreased in all metformin-treated cell lines. The expression of AKT mRNA was also reduced by metformin in both SW1736 and 8305C cells but did not alter in the C643 cell line. We also considered the metformin effect on FOXO1 expression on these cells; metformin markedly decreased the mRNA levels of FOXO1 in all examined cell lines. Given that the PI3K and AKT proteines are actived in the phosphorylated form and FOXO1 is inactive in this form, metformin effects were investigated on phosphorylation and subsequently their activation by ELISA method, according to the results, metformin had no significant effect on these protein phosphorylation levels in all metformin-treated ATC cell lines. In a study performed by Liu et al. [17], metformin inhibited cutaneous squamous cell carcinoma (SCC) prolifreration by decreasing PI3K and p-AKT levels and downregulating of PI3K mRNA levels without changes in AKT mRNA level they concluded that metformin-inhibited proliferation of SCC cells might be associated with changes in phosphorylation levels of the PI3K/AKT signaling pathway. It was demonstrated that metformin inhibites the growth of breast cancer cells via the activation of AMPK and consequently the inhibition of AKT [8]. In another study, Yung *et al* reported that the activation of AMPK by metformin caused downregulation of p-AKT, p-FOXO3a and phosphorylated forkhead box M1 (p-FOXM1) in the cervical cancer cell line. In this study, the expression of FOXM1 was not significantly altered by metformin after the inhibition of FOXO3a by siRNA, indicating that metformin may suppress cervical cancer cell growth via AMPK activation and AKT/FOXO3a/FOXM1 inhibition [9]. Similar to Zakikhani, Karnevi *et al* revealed that the activation of AMPK and suppression of the phosphorylation of AKT by metformin had anti-proliferative effect on pancreatic cancer cells [32]. Unlike the results mentioned regarding metformin decreases the level of p-AKT, Sarfstein et al. demonstrated that metformin did not decrease AKT phosphorylation in human endometroid endometrial carcinoma cells (ECC-1 and Ishikawa; Type I) and ECC-1 cells; they attributed these paradoxical results to the mutations in the *RAS* proto-oncogene, p53 and *PTEN* tumor suppressor gene, leading to continuous phosphorylation of AKT [33]. Thus, according to our findings it can be speculated that metformin significantly suppreses ATC cell lines proliferation by downregulating mRNA expression of PI3K and AKT in the PI3K/AKT signaling pathway without effecting PI3K and AKT phosphorylation in these cell lines. Until now, there is a lack of significant evidence on the effects of metformin on FOXO family members. In two separate studies, Sarfstein [33] and Song [34] demonstrated that metformin reduced lipid accumulation by decreasing FOXO1 levels in both uterine serous carcinoma (USC) cells and macrophages, respectively. In two other separate studies, Li [35] and Zatara [36] showed that metformin decreased nuclear entry rate and recruitment of FOXO1; they also illustrated that metformin repressed the activities of FOXO1 via reducing its nuclear entry rate in a manner dependent on AMPK activation. Their finding is in accordance to us regarding the effects of metformin on FOXO1 mRNA expression on cancerous cell lines. Compared to the above Barbato et al. reported contradictory findings on the effects of metformin on FOXO1 [37]; they showed that metformin increased lysosomal acid lipase (Lipa) in adipocytes, which was associated with FOXO1 upregulation and its nuclear translocation.

According to previous studies and also our findings, metformin decreases FOXO1 expression in different cells and ATC cells, indicating apparently a contradiction as metformin, on the one hand, acts as an antitumor and antiapoptosis agent, but on the other hand apparently downregulates

FOXO1 expression as a potential tumor suppressor factor with important roles in cell proliferation and apoptosis. It can be speculated that the metformin AMPK-stimulated anti-proliferative effects are stronger than its inhibitory effect on FOXO1 expression as a tumor suppressor factor such that the first effect mentioned can compensate for the second inhibitory effect.

In conclusion, our study demonstareted that metformin decreased cell viability in ATC-derived cells *ex vivo* in a dose-and time-dependent manner, which was significantly associated with the downregulation of PI3K and AKT mRNA levels of the PI3K/AKT signaling pathway. FOXO1 is one of the targets of PI3K/AKT involved in a variety of cellular processes. It has been reported that FOXO1 may play an important role in the development of thyroid cancer. Our results showed decrease in FOXO1 expression of the ATC cells which was intensified by metformin. However apparently, metformin exerts its anticancer effects via another stronger pathway (AMPK pathway), because of which we did not observe increase in cell proliferation following FOXO1 down-regulation by metformin.

Based on studies investigating metformin effects on FOXO1, it seems that other factors may be involved in PI3K, AKT and FOXO1 expression regulation which affected by metformin. However, the exact molecular mechanism of the metformin on the inhibition of PI3K/AKT pathway and subseqent decrease of cell viability has remained unclear and further studes are needed for its clarification.

Conflict of interest

There is no conflict of interest.

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Table 1. Primer sequences for reverse transcription-PCR (RT-PCR).

Target Gene	*Sequence (5'→3')*	*Product Size (bp)*
β-Actin	***F:*** GATCAAGATCATTGCTCCTCCT ***R:*** TACTCCTGCTTGCTGATCCA	108
AKT-1	***F:*** CACTTTCGGCAAGGTGATCC ***R:*** GTCCTTGGCCACGATGACTT	94
PI3K	***F:*** CAGAACAATGCCTCCACGA ***R:*** CACGGAGGCATTCTAAAGTC	122
FOXO1	***F:*** AACTACAGCCAAAATCACTG ***R:*** ATGACAGGATTTCAACACAC	129

Table 2. IC_{50} values of metformin. Values are shown as Mean ± SD for three independent examinations.

Cell Line	*IC50 (Mean ± SD*)*		
	24h	***48h***	***72h***
SW1736	53.6 ± 3.1	26.6 ± 0.9	21.5 ± 1.1
C-643	58.7 ± 2.4	30.3 ± 3.0	18.4 ± 1.5
8305C	92.1 ± 6.8	46.8 ± 1.9	36.8 ± 1.5

*SD: Standard Deviation

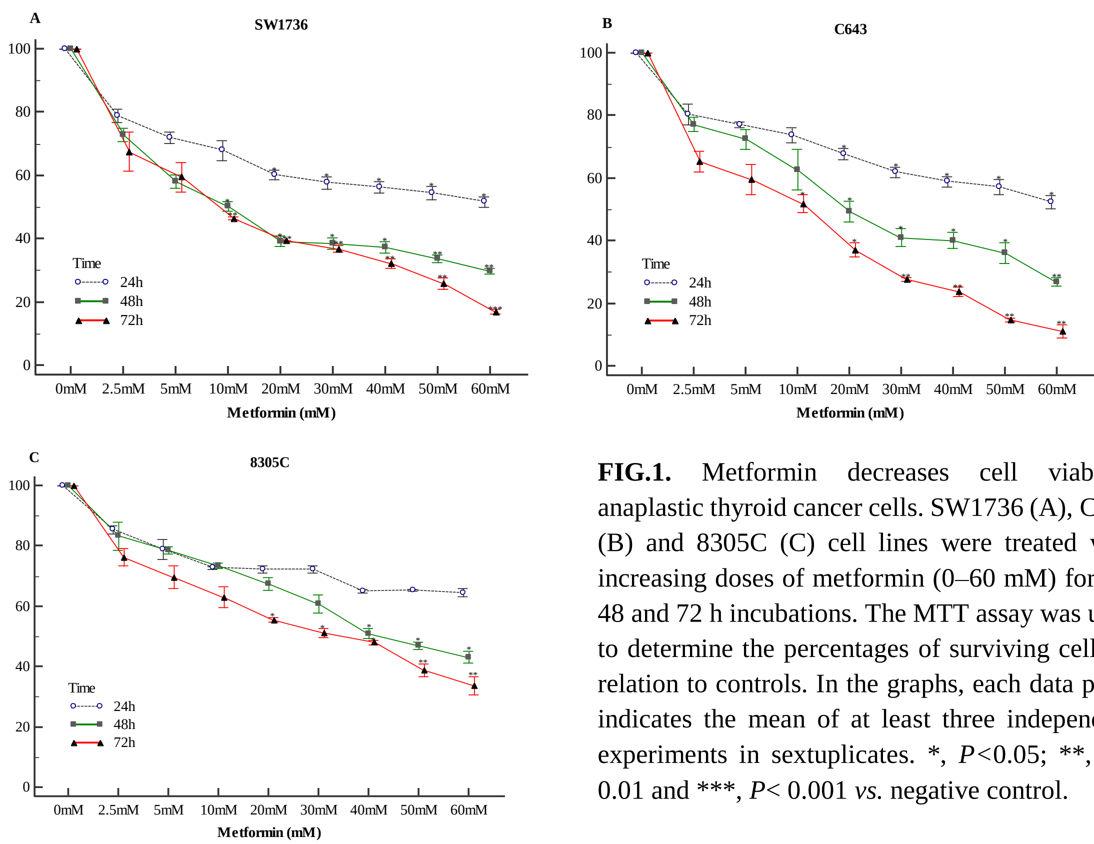


FIG.1. Metformin decreases cell viability anaplastic thyroid cancer cells. SW1736 (A), C643 (B) and 8305C (C) cell lines were treated with increasing doses of metformin (0–60 mM) for 24, 48 and 72 h incubations. The MTT assay was used to determine the percentages of surviving cells in relation to controls. In the graphs, each data point indicates the mean of at least three independent experiments in sextuplicates. *, *P*<0.05; **, *P*< 0.01 and ***, *P*< 0.001 *vs.* negative control.

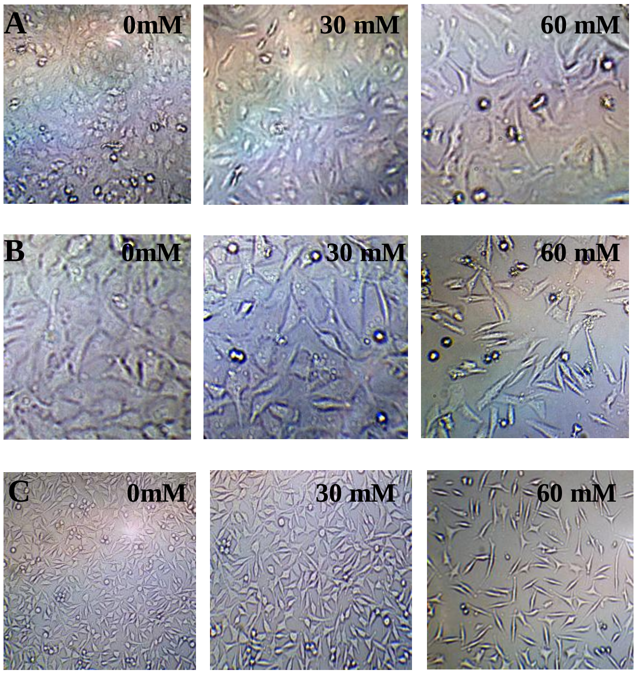
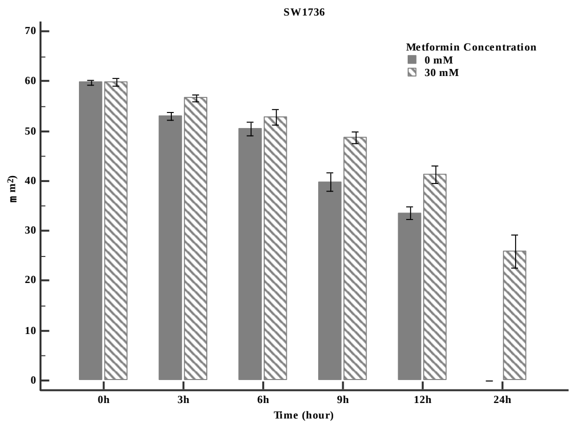
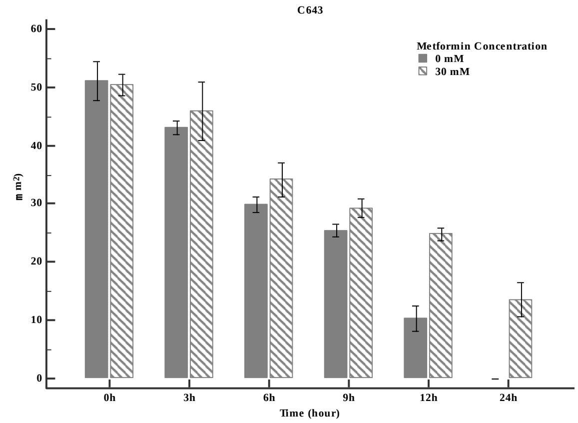


FIG.2. Morphology of human ATC cell lines after treatment with different concentrations of metformin for 48 h (10 magnification). *Panel A:* SW1736, *Panel B:* C643 and *Panel C:* 8305C.





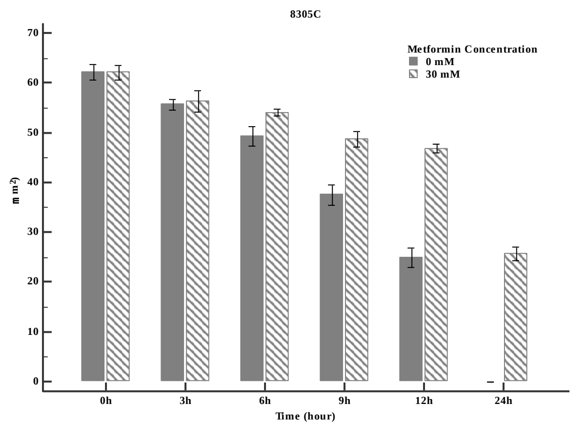


FIG.3. Cell migration assay following treatment with metformin. SW1736, C643 and 8305C cell lines were grown in 24-well plates. After 24h the culture medium was replaced by fresh medium containing 0 and 30mM metformin. Snapshots were taken in 0-24h time range. Reduced cell migration was observed in metformin treated cells. *P<0.05*

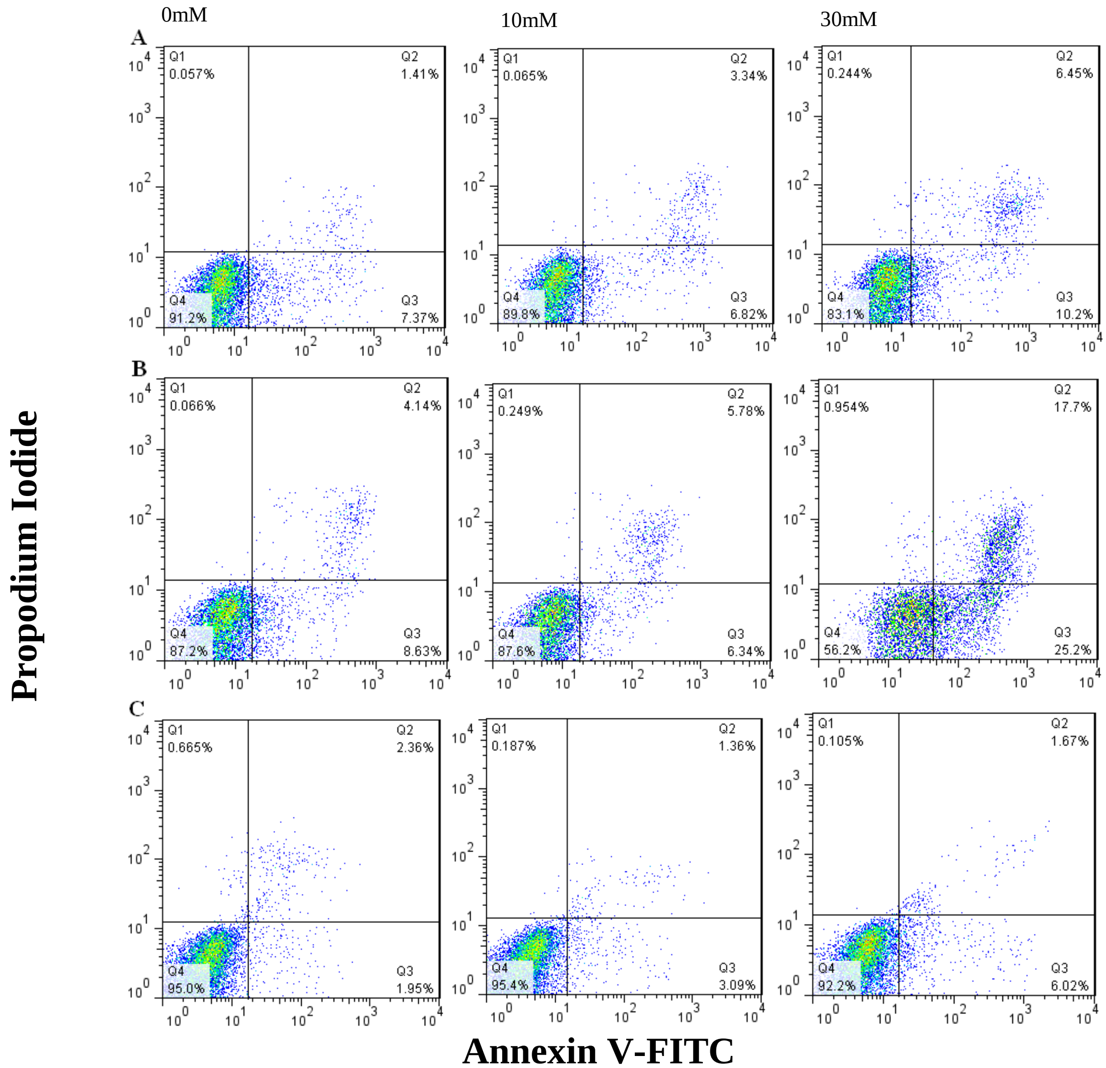


FIG.4. Apoptosis of SW1736 (*row A dot plots*), C643 (*row B dot plots*) and 8305C (*row C dot plots*) cells in response to different concentrations of metformin (0, 10 and 30 mM) for 48h were determined by Annexin V/PI staining. The percentage of early apoptosis (*lower right quadrangle*) and late apoptosis/necrosis (*upper right quadrangle*) were calculated by Flowjo software.

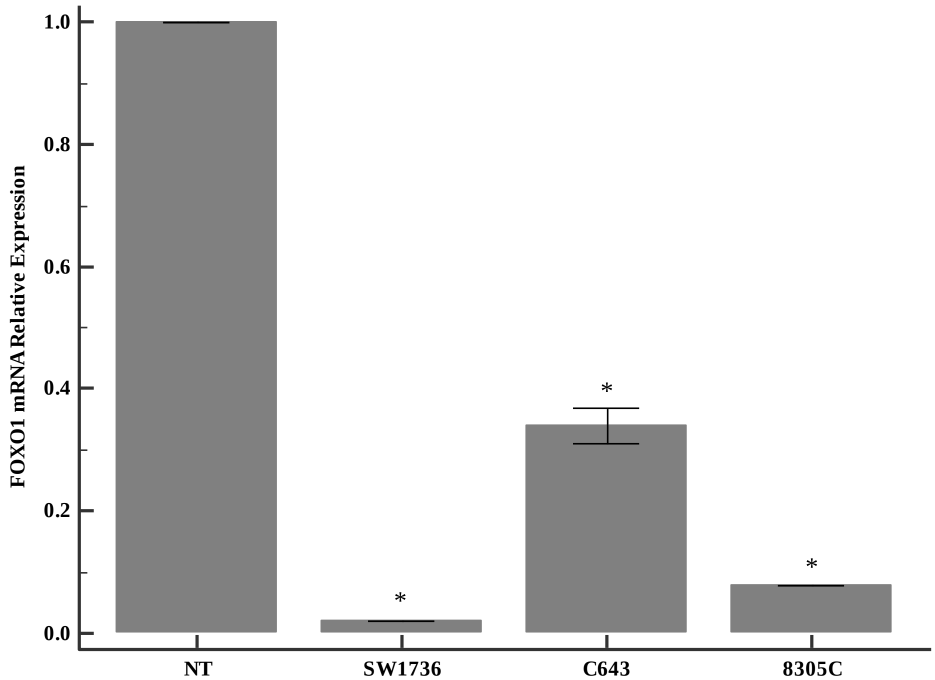


FIG.5. FOXO1 expression decreased in anaplastic thyroid cancer cell lines compared with human normal thyroid tissue. Results are the mean ± SD of three independent experiments performed in triplicate and changes with respect to the mRNA levels of normal cells (considered significant at $P < 0.05$). NT: normal tissue.

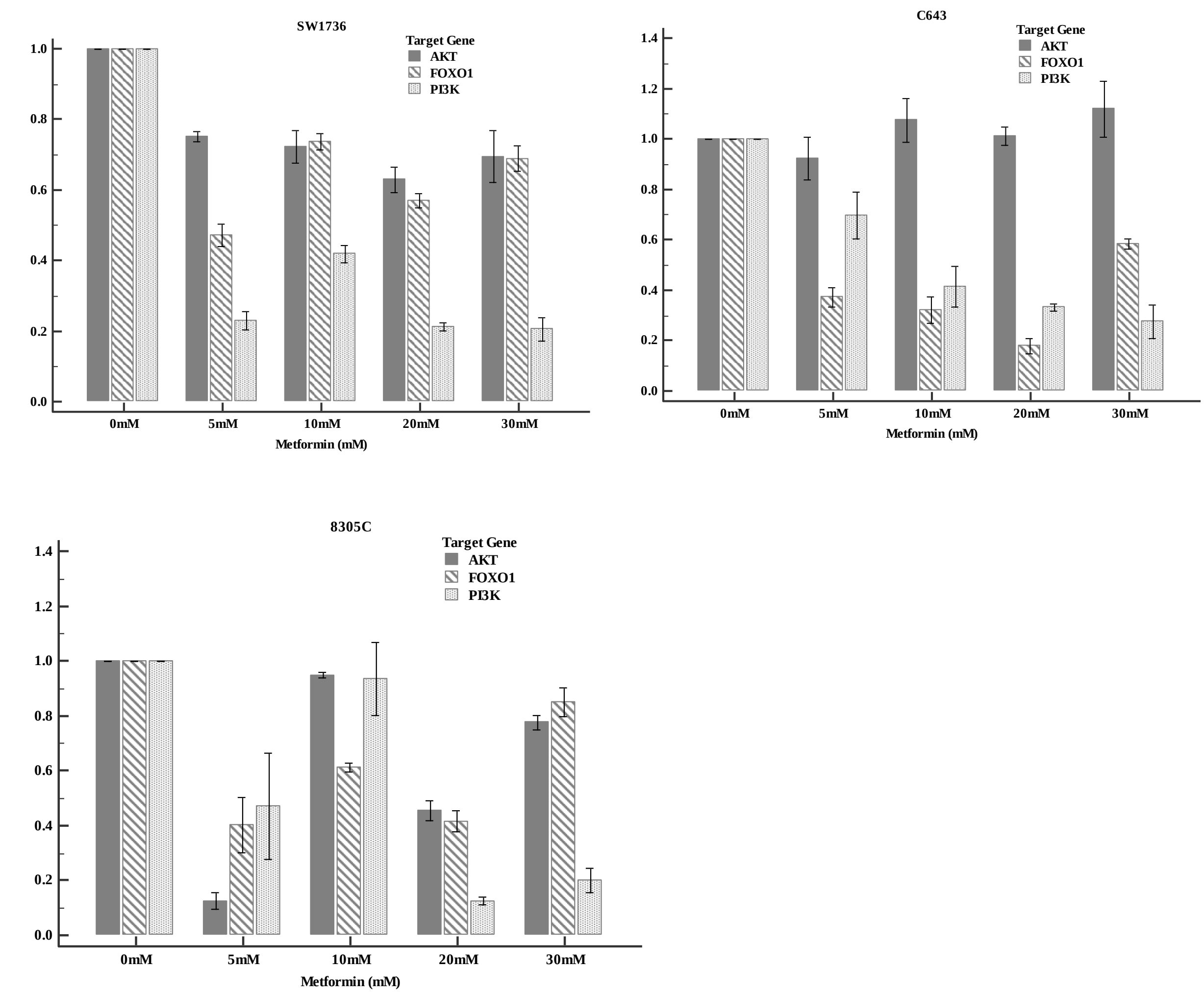
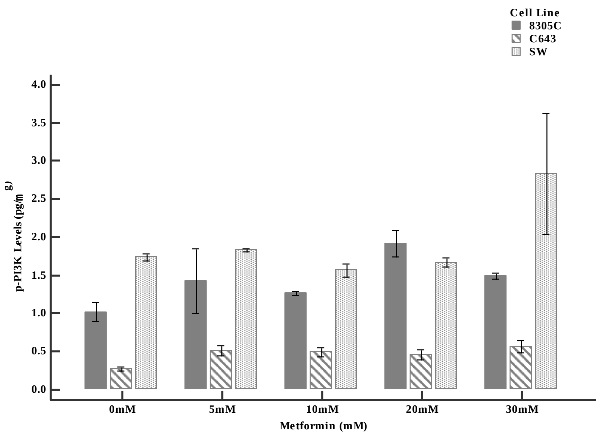
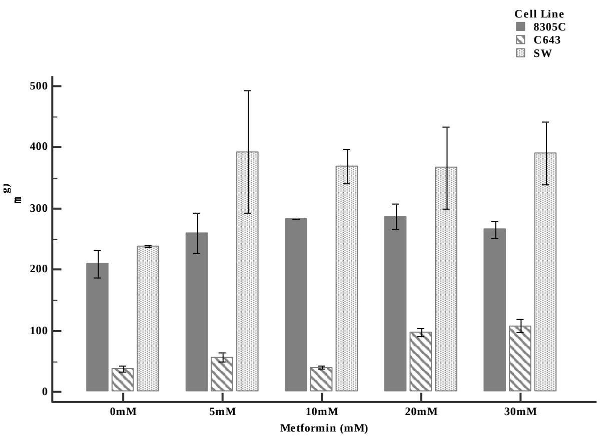


FIG.6. Quantitative Real-Time PCR analyses revealed that the expression of *PI3K* mRNA was decreased in metformin-treated SW1736, C643 and 8305C cell lines compared with negative control. The expression of AKT mRNA was also decreased in SW1736 and 8305C cell lines whereas no change was observed in its expression in C643. FOXO1 mRNA expression was also decreased in all SW1736, C643 and 8305C cell ines. Data were presented as means ± SEM (n = 3). $P < 0.05$.





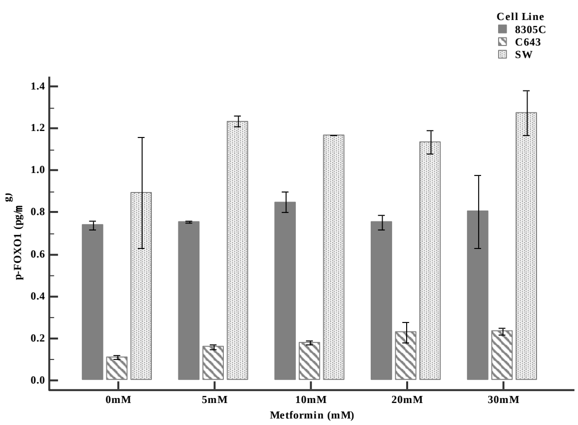


FIG.7. Enzyme-linked Immunoassay analyses revealed that there was not a significant change in p-PI3K, p-AKT and p-FOXO1 levels in ATC cell lines following the treatment with metformin compared with untreated groups of cells. *P>0.05*